Pathol. Oncol. Res. (2014) 20:113–117
DOI 10.1007/s12253-013-9669-2

RESEARCH

The Relationship Between Gastric Cancer and *Helicobacter Pylori* in Formaldehyde Fixed Paraffin Embedded Gastric Tissues of Gastric Cancer Patients-Scorpion Real-Time PCR Assay Findings

Taghi Naserpour Farivar · Pouran Johari · Reza Najafipour · Amir Farzam · Neda Nasirian · Fatemeh HajManouchehri · Hassan Jahani Hashemi · Akram Azimi · Mohammad Bahrami

Received: 7 December 2012 / Accepted: 26 June 2013 / Published online: 1 November 2013

Abstract Gastric cancer is the second leading cause of cancer-related deaths worldwide and it seems that environmental and lifestyle factors and infection with *Helicobacter pylori* (*H.pylori*) have had a major role in the etiology of gastric cancer. The aim of this study was to investigate the presence of *H. pylori* DNA in archival gastric tissues of patients with gastric cancer disease by rapid, sensitive and specific technique of Scorpion Realtime PCR. This retrospective cross-sectional study was performed on 285 paraffin embedded gastric specimens of patients who were pathologically proved for gastric cancer admitted in Bou-Ali, Shahid Rajaie and Dehkhoda hospitals and Bahar and Farzam private laboratory in Qazvin city in Iran during 2009 and 150 paraffin embedded pathological specimens of patients with other proved diagnosis other than gastric cancer. Results of our Scorpion Realtime PCR analysis showed that DNA of *H. pylori* DNA was present in 78.42 % of our total specimens. Modified McMullen's Staining of paraffin embedded sections was positive in 210 patients. Also we were not able to finding significant relationship between demographic characteristics of our studied patients and presence of *H. pylori* DNA in their formaldehyde fixed paraffin embedded gastric tissues samples. Existence of *H. pylori* in gastric tissue samples of patients with gastric cancer is controversial and our results indicated that in our studied specimens prevalence of *H.pylori* was significantly more than recent published reports.

Keywords Scorpion · Real-time · *Helicobacter pylori* · Gastric · Cancer

T. Naserpour Farivar · P. Johari · R. Najafipour (✉) · A. Farzam · N. Nasirian · F. HajManouchehri · H. Jahani Hashemi · A. Azimi · M. Bahrami
Cellular and Molecular Reasearch Center, Qazvin University of Medical Sciences, Qazvin, Qazvin, Iran
e-mail: rnajafipour@gmail.com

T. Naserpour Farivar
e-mail: t.naserpour@qums.ac.ir

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide. International Agency for Research on Cancer reported that in Asia malignancy of the stomach is the most common cancer and nearly two-thirds of it occurs in developing countries [1]. Although the incidence and mortality of distal gastric cancers has decreased in the Western world, the incidence of proximal tumors is increasing in the male population of those regions. In contrast, in Asian continent distal tumors have increased while proximal tumors have decreased [2]. This geographic variation accompanying with differences in lifestyle, time trends and migratory effects associated with the incidence of gastric cancer suggest that environmental and lifestyle factors have a major role in the etiology of gastric cancer [3, 4].

Gastric cancer is an outcome of a complex interaction between host and environmental factors and *H. pylori* infection. Scientific evidence suggests the importance of host factors and microbial agents in pathogenesis of cancer [5, 6]. *Helicobacter pylori* (*H. pylori*) are Gram-negative, microaerophil and spiral-shaped bacteria. *H. pylori* infection affects 50 % of the population, worldwide [7]. *H. pylori* infection initiate an inflammatory response which is influenced by genetic polymorphisms which in turn can either accentuate

or attenuate the host's response to inflammation and affecting the interaction's outcome. Recent advances in the study of the etiological backgrounds of non-cardia gastric adenocarcinoma revealed that *H. pylori* infection is an established important causal factor for this kind of cancer.

In the process of gastric carcinogenesis, bacterial virulence factors that have been implicated include the cytotoxin-associated gene A antigen (Cag A), vacuolating cytotoxin (VacA), and outer membrane proteins (OMP) [8]. The *cagE* genotype has been associated with gastric cancer in some studies, but contrary results have also been published [9, 10].

On the basis of these differences of opinion about the prevalence of H. pylori association with gastric cancer exists in the literature [11–17] we planned this study to determine the overall prevalence of *H. pylori* DNA in gastric carcinoma.

Materials and Methods

Patients and Methods This retrospective cross-sectional study was performed on 285 paraffin embedded gastric specimens of patients who were pathologically proved for gastric cancer admitted in Bou-Ali, Shahid Rajaie and Dehkhoda hospitals and Bahar and Farzam private laboratory in Qazvin city in Iran during 2009. Ethical Committee of Qazvin University of Medical Sciences approved our study. Control samples were 150 paraffin embedded pathological specimens of patients with other proved diagnosis other than gastric cancer.

Clinical Samples Initially, the paraffin embedded gastric tissues were sectioned into 50 μm slices followed by DNA extraction using DNA preparation protocol obtained from Section of Cancer Genomics, Genetics Branch, NCI, Institute of Health.

Modified McMullen's Staining of Gastric Biopsy The paraffin embedded sections was dewaxed, dehydrated and covered by carbol-fuchsin for 2 min. The sections rinsed and stained malachite green for 2 min. Then slides rinsed in tap water, air dried and observed under the light microscope. Magenta color particles are indicative of *H. pylori*.

Scorpion Real-Time Scorpion real-time PCR was performed using ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA). The tails of the Scorpion primers were designed as previously described by Burucoa [18]. All primers and probes used were synthesized by Metabion company (Germany) (Table 1). The total volume of the real-time PCR was 20 μl containing 5 μl of DNA from a clinical sample or bacterial isolate, 0.1 μM of oligonucleotide primer 23SF2 and 0.08 μM of 23SScWT, while the total volume of 20 μl was achieved by addition of distilled water. The cycling conditions were adjusted in a way suitable to be used by ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA) with an initial denaturation at 95 °C for 45 s, 50 cycles at 95 °C for 15 s, 55 °C for 34 s, and 72 °C for 20 s. The acquisition of a signal was performed at 57 °C during each cycle. A negative control for Scorpion real-time PCR obtained by observation of no amplification signal except internal amplification by adding DDW instead of DNA to the prepared real-time PCR master mix. A positive control for Scorpion PCR was constituted by using extracted DNA from H. pylori ATCC 26695. Positive extraction control was performed for each biopsy specimen in a separate tube in a final volume of 25 μl with Premix Ex Taq (TaKaRa, Shiga, Japan), 5 μl of extracted DNA from biopsy specimen, 0.25 μM of BGLO1 and BGLO2 primers, and 0.5× Sybergreen 1 (Sigma Aldrich) and on the basis of the results of this test, 285 of the collected biopsies were entered in our study. The cycling program was 1 cycle at 95 °C for 10 s and 40 cycles of 95 °C for 5 s, 55 °C for 34 s, and 72 °C for 10 s.

Statistical Analysis Data were analyzed using SPSS11.5 software with 95 % confidence intervals (95 % CI).

Results

In this survey the prevalence of *H. pylori* infection in gastric samples of patients with gastric cancer was determined using the data obtained by:

Detection of H pylori by Scorpion Real-Time PCR: Initially, Scorpion real-time PCR was performed on *H. pylori* ATCC 26695. Of total 285 samples, 224 biopsies found to be positive for *H. pylori* by Scorpion real-time PCR (78.49 %) whereas 61 samples showed negative results. Among samples with positive Scorpion real-

Table 1 Primers and probes sequences for detection of *H.pylori* in pathological specimens of gastric patients and controls

Oligonucleotide	Sequence
23SScWT	5′-FAM-AAGGTAGGTGAAAATTCCTCCTACC BHQ1 HEG GGACCACGGGGTCTTT-3′
23SF2	5′-TGCGAACTGTGTGTTCACTAGC-3′
BGLO1	5′-ACACAACTGTGTGTTCACTAGC-3′
BGLO2	5′-CAACTTCATCCACGTTCACC-3′

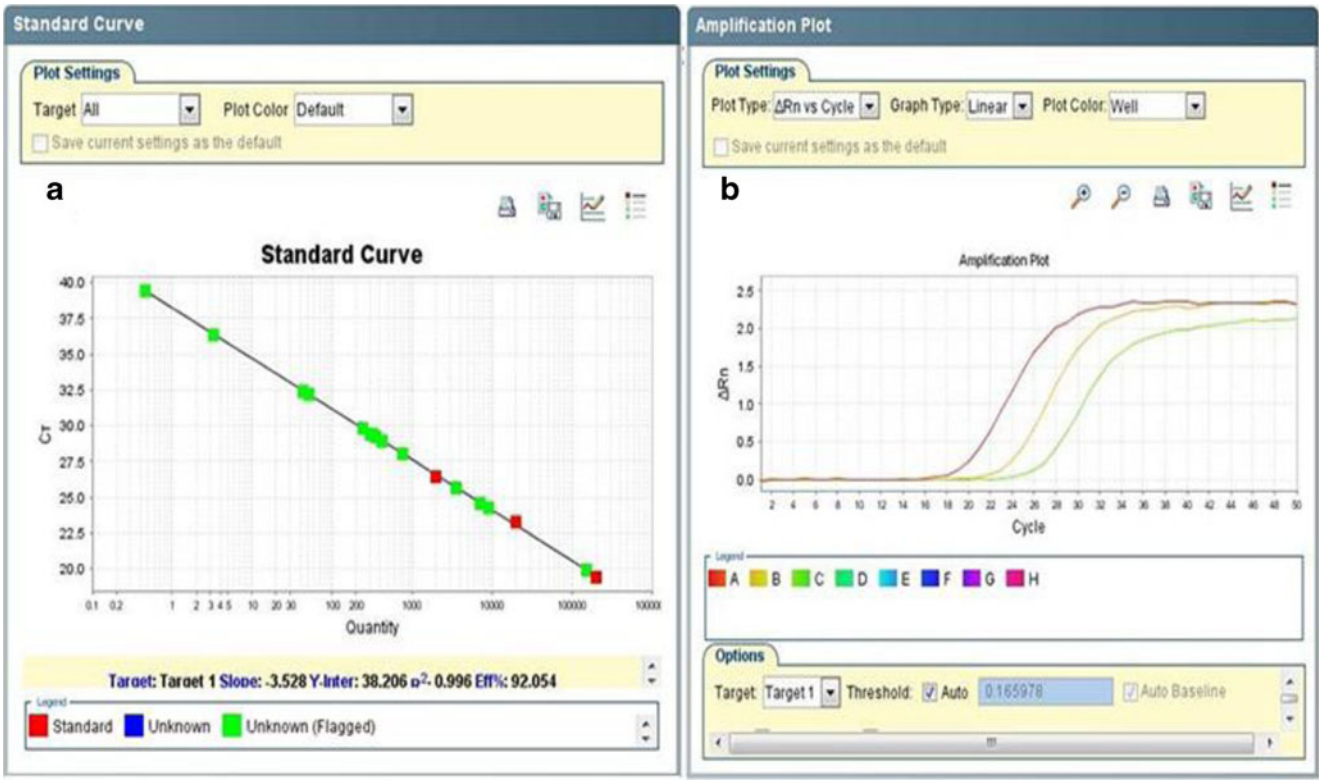

Fig. 1 **a** Standard curve used to calculate the concentration of *H.pylori* DNA in unknown samples. The linear regression coefficient is 0.996 and efficiency of PCR is 92.054 %; **b** Fluorescent curves of the standard dilution series. From the left to the right 10^4, 10^3, 10^2 and the negative control is presented by the horizontal curve

time PCR test, 210 samples were also positive with Modified McMullen's Staining (93.75 %). From of total 150 control samples 47 biopsies found to be positive for *H. pylori* by Scorpion real-time PCR 47 (31.3 %) whereas 103 samples showed negative results. Among samples with positive Scorpion real-time PCR test, 40(85.1 %) samples were also positive with Modified McMullen's Staining.

Scorpion Real-Time PCR: Scorpion real-time PCR on DNA extracts of *H. pylori* ATCC 26695 as control strains produced expected signals. The standard curve used to calculate the concentration of *H. pylori* DNA in unknown samples is presented in Fig. 1a. The linear regression coefficient was 0.996 and the efficiency of PCR 92.054 %. Figure 1b shows the fluorescent curves of the standard serial dilution from 1×10^4 to 1×10^2 copy per microliter. Electrophoresis pattern of products in scorpion real-time PCR assay in agarose gel is shown in Fig. 2. A 140 bp fragment in the peptidytransferase gene of 23S rRNA showed a successful amplification. Of total patients (285 cases) with gastric cancer who entered the study, 224 cases (78.24 %) were found to have *H. pylori* DNA in their gastric biopsy specimens by Scorpion real-time PCR method. Our study showed that there is no significant association between demographic characteristics of our patients, history of gastric involvement, gastric disease, and the presence of bacterium. Also we were not able to find any relationship between the sex, family income, and accommodation variables and the colonization of *H. pylori* in gastric cancer tissues (data not shown).

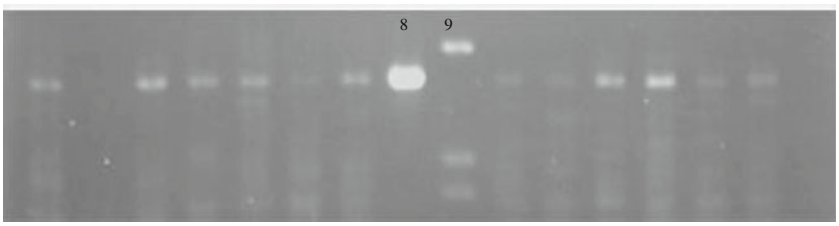

Fig. 2 The Scorpion Realtime PCR products of tested samples. PCR products were electrophoresed in a 2 % agarose gel. Lane 8: Helicobacter pyori ATCC26695 with a positive 140 bp band; Lane 9:100 bp DNA ladder. Other lines: Scorpion PCR products of samples

Discussion

H. pylori infects human in childhood and at this time it has infected one half of the world's population. It is the only bacterium which is listed in the microbial agents which are involved in human cancer diseases.

Our finding showed that from the total of 285 formaldehyde fixed paraffin embedded samples of pathologically proved gastric cancer patients which enterd to our study, 224 patients had *H. pylori* DNA while Hashemi and colleagues reported that in their 161 studied normal mocusa specimens of Iranian population 54 had *H.pylori* (33.5 %) [19].

The results of published researches in the medical literature related to the involvement of *H. pylori* in gastric cancer is controversial and confirmation of this involvement and determination of the level of this involvement in different societies are also important issues.

As *H. pylori* is a fastidious bacteria, microbiological methods of culture and identification of these bacteria are difficult and needs a long time and adequate sensitivity and specificity of modern molecular methods in diagnosis of these bacteria make them attractive methods for studying prevalence of *H. pylori* in different clinical specimens [6].

Results of our study showed that from total 224 number of specimens with *H.pylori* DNA, all had CagA positive genotype (Data not shown).

Khanna et al. in a study on 50 proved cases of gastric cancer found that the prevalence rate of *H. pylori* infection in their patients was lower than in the control population, suggesting that *H. pylori* may not be responsible for gastric carcinogenesis in this population [16]. In other study Rudi and colleagues in their study on the sera of 111 Caucasian patients with histologically confirmed gastric cancer found that their data do not provide evidence that the contribution of *H. pylori* infection to the carcinogenesis of gastric cancer is of major significance at least in a population with low gastric cancer rates and with high socioeconomic status [17].

On the other hand Babus and colleagues in a study on analysis of 12 prospective case—control studies concluded that between about 65 % and 80 % of non-cardia gastric cancers were attributable to *H. pylori* infection [13, 19]. Uemura et al. studied 1,526 Japanese patients, of whom 1,246 had *H. pylori* infection and 280 were not infected. Over a mean follow-up period of 7.8 years, gastric cancer developed in 2.9 % of patients with *H. pylori* infection and none of the uninfected patients developed gastric cancer, giving a relative risk of 34.5 [14].

Martínez and colleague in a case-control study including 46 gastric cancer cases and 99 controls with non-atrophic gastritis from a high risk zone for gastric cancer showed that there is a positive association between *Helicobacter pylori* CagA positive strains with non-cardial gastric cancer etiology [15].

Although we studied a relatively large population and we used sensitive and specific method of scorpion real time PCR assay, our results indicated that in our studied specimens prevalence of *H.pylori* was many times more than recent published reports [20, 21] and is in consistent with the latter studies indicating a possible role for *H. pylori* in gastric cancer and observed higher rate of prevalence of these bacteria in our patients, may lay back in the polymorphism and genetic diversity of isolated *H.pylori* strains which influence the oncogenic potential of these bacteria [22]

Acknowledgment The authors wish to thanks Deputy of Research in Qazvin University of Medical Sciences for Financial support of this study.

References

1. Ferlay J, Bray F, Pisani P (2001) GLOBOCAN 2000: Cancer incidence, mortality and prevalence worldwide, version 1.0. IARC Cancer Base No 5. IARC Pres, Lyon, 1
2. Crew KD, Neuget AI (2006) Epidemiology of gastric cancer. World J Gastroenterol 12:354–363
3. Tiwari SK, Manoj G, Vasanth Kumar G, Sivaram G, Hassan SI (2008) Prognostic significance of genotyping Helicobacter pylori infection in patients in younger age groups with gastric cancer. Postgrad Med J 84:193–197
4. Ries LAG, Kosary CL, Hankey BF (1994) International Agency for Research on Cancer Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, liver flukes, and Helicobacter pylori. International Agency for Research on Cancer, Lyon, pp 177–240
5. Fox JG, Wang TC (2007) Inflammation, atrophy, and gastric cancer. J Clin Invest 117:60–69
6. Naserpour Farivar T, Pahlevan AK, Johari P (2012) Assessment of Helicobacter pylori prevalence by scorpion Real-time PCR in chronic tonsillitis patients. J Global Infec Dis 4:38–42
7. Correa P, Piazuelo MB (2008) Natural history of Helicobacter pylori infection. Dig Liver Dis 40:490–496
8. McNamara D, El-Omar E (2008) Helicobacter pylori infection and the pathogenesis of gastric cancer: a paradigm for host-bacterial interactions. Dig Liver Dis 40:504–509
9. Fock KM, Dhamodaran S, Teo EK (2006) Is H. pylori genotypes significant in Asian patients with gastric cancer and GERD. Gut 55(Suppl. V):A258
10. Erzin Y, Koksal V, Altun S (2006) Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA, babA2 genotypes and correlation with clinical outcome in Turkish patients with dyspepsia. Helicobacter 11:574–580
11. Chomvarin C, Namwat W, Chaicumpar K (2008) Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA and babA2 genotypes in Thai dyspeptic patients. Int J Infect Dis 12:30–36
12. Ghasemi A, Shirazi MH, Ranjbar R (2008) The prevalence of cagA and cagE genes in Helicobacter pylori strains isolated from different patient groups by polymerase chain reaction. Pak J Biol Sci 11:2579–2583
13. Babus V, Strnad M, Presecki V (1998) Helicobacter pylori and gastric cancer in Croatia. Cancer Lett 125:9–15
14. Uemura N, Okamoto S, Yamamoto S (2001) Helicobacter pylori infection and the development of gastric cancer. N Engl J Med 345:784–789

15. Martínez T, Hernández-Suárez G, Mercedes Bravo M, Trujillo E, Quiroga A, Albis R (2011) Association of interleukin-1 genetic polymorphism and CagA positive Helicobacter pylori with gastric cancer in Colombia]. Rev Med Chil 139:1313–1321
16. Khanna AK, Seth P, Nath G (2002) Correlation of Helicobacter pylori and gastric carcinoma. J Postgrad Med 48:27–35
17. Rudi J, Müller M, von Herbay A (1995) Lack of association of Helicobacter pylori seroprevalence and gastric cancer in a population with low gastric cancer incidence. Scand J Gastroenterol 30:958–963
18. Burucoa C, Garnier M, Silvain C, Fauchère JL (2008) Quadruplex real-time pcr assay using allele-specific scorpion primers for detection of mutations conferring clarithromycin resistance to helicobacter pylori. J Clin Microbiol 46:2320–2326
19. Hashemi MR, Rahnavardi M, Bikdeli B, Dehghani Zahedani M (2006) H pylori infection among 1000 southern Iranian dyspeptic patients. World J Gastroenterol 12(34):5479–5482
20. Bauer B, Meyer TF (2011) The human gastric pathogen helicobacter pylori and its association with gastric cancer and ulcer disease. Ulcers 2011, Article ID: 40157, 23 pages
21. McColl K (2010) Helicobacter pylori infection. N Engl J Med 362:1597–1604
22. Fuccio L, Eusebi LH, Bazzoli F (2010) Gastric cancer, Helicobacter pylori infection and other risk factors. World J Gastrointest Oncol 2:342–347